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(71) Applicant: **ELIM BIOPHARMACEUTICALS, INC.** [US/US]; 901 Grandview Drive, South San Francisco, CA 94080 (US).

(72) Inventors: **LU, Xianbin**; 311 Faxon Avenue, San Francisco, CA 94112 (US). **SUN, Lihyun**; 140 Ware Road, Woodside, CA 94062 (US). **ZHANG, Yilin**; 140 Ware Road, Woodside, CA 94062 (US).

(74) Agents: **CHOW, Y., Ping** et al.; Heller Ehrman White & McAuliffe LLP, 275 Middlefield Road, Menlo Park, CA 94025-3506 (US).

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(54) Title: NOVEL PLASMID DNA VECTORS

(57) Abstract: This invention relates to plasmid backbones and constructs wherein non-essential nucleotide sequences have been substantially removed. These backbones can be used to clone genes of interest. The constructs are useful in gene therapy, DNA vaccines and the *in vitro* production of polypeptides and/or proteins.

Novel Plasmid DNA Vectors

Field

The present invention relates generally to the field of plasmid constructs, specifically to constructs that are substantially free of non-essential sequences. The plasmids are useful for delivery of therapeutic nucleic acids to cells. In addition, this invention relates to methods of using those constructs as well as methods for selection of desired plasmids. Such constructs and methods are useful in most molecular biology applications, such as gene therapy, DNA vaccines and the *in vitro* production of polypeptides and/or proteins.

Background

Transgenesis refers to the process of introducing exogenous genes into the germ line of an organism. Transgenesis of humans would allow for the elimination of disease genes in a population of offspring, however, technical as well as ethical issues likely will prevent any transgenic experiments to be carried out with human eggs. Therefore, the ability to replace known disease genes with normal copies in afflicted humans is the ultimate goal of gene therapy. Human gene therapy protocols aim to introduce correcting copies of disease genes into somatic cells of the affected individual. Expression of a correct copy of an affected gene in somatic cells avoids transmission through the germ line, thereby, avoiding many of the ethical issues of transgenesis.

In the late 1980s, gene therapy was searching for a viable vector—a virus or lipid vehicle that would deliver DNA to cells in a way that would promote uptake and expression of the DNA. During the search it was discovered that injection of DNA plasmids in saline solution alone—that is, without the use of a vehicle—stimulated expression of foreign proteins in mice. The amounts of protein made by tissue receiving this so-called "naked DNA" were too low to correct an enzyme defect or provoke destruction of a tumor. However, the levels

were high enough to stimulate an immune response. DNA vaccination involves the deliberate introduction into cells of a DNA plasmid carrying an antigen-coding gene that induces an immune response.

The administration of plasmid DNA expression vectors causes intracellular synthesis of the encoded proteins and induction of long-lasting cellular and humoral immune responses. The exogenous DNA it must cross not only the cellular membrane but also the nuclear membrane since the transcription machinery is inside of the nucleus. The nuclear membrane has "pores," about 10 nm in diameter, through which molecules pass. For example, the nuclear proteins are synthesized in the cytoplasm and must enter the nucleus to transcribe the DNA to mRNA. Similarly, the mRNA must pass out of the nucleus to the cytoplasm to be translated. As indicated above, the nuclear pores are very small. For a given vector to cross membranes or pass through nuclear pores, a smaller plasmid would presumably encounter fewer obstacles during transfection. Thus, small sized vectors are desirable.

Plasmids are an essential element in genetic engineering and gene therapy. Plasmids are circular DNA molecules that can be introduced or transfected into bacterial cells by transformation which replicate autonomously in the cell. They offer several advantages as vectors, the most important of which is the ability to confer antibiotic resistance to the host cell. This allows direct selection for cells that receive and maintain recombinant DNA plasmids. Plasmids also allow the amplification of cloned DNA. Some plasmids are present in 20 to 50 copies during cell growth, and after the arrest of protein synthesis, as many as 1000 copies per cell of a plasmid can be generated.

Plasmid design and construction utilizes several key factors. First, plasmid replication origins determine plasmid copy number, which affects production yields. Plasmids that replicate to higher copy number can increase plasmid yield from a given volume of culture, but excessive copy number can be deleterious to the bacteria and lead to undesirable effects. Artificially constructed plasmids are

sometimes unstably maintained, leading to accumulation of plasmid-free cells and reduced production yields.

To overcome this, genes that code for antibiotic resistance phenotype are included on the plasmid. Antibiotics are often added to kill or inhibit plasmid-free cells. Most general-purpose cloning vectors contain ampicillin resistance (β-lactamase) genes while others have used the neo gene that encodes resistance to kanamycin and neomycin. Use of these genes can be problematic because of the potential for residual antibiotic in the purified DNA, which can cause an allergic reaction in a treated patient, as well as the potential for the transfer of antibiotic resistance genes to a potential pathogen.

Other plasmid elements that have been studied include partition elements. Such elements help stabilize plasmid maintenance independent of antibiotic selection. In addition, other elements promote monomerization of the plasmid. Some plasmids are prone to forming dimers, trimers and higher multimers that can reduce yield and interfere with maintenance, as well as generating a more complicated product profile. Multimer resolution elements have been employed to promote monomerization of plasmids.

In addition to elements that affect the behavior of the plasmid in the host bacteria, such as E. coli, plasmid vectors have also been shown to affect transfection and expression in eukaryotic cells. Certain plasmid sequences have been shown to reduce expression of eukaryotic genes in eukaryotic cells when carried in cis. Plasmid sequences also have been shown to fortuitously contain binding sites for transcriptional control proteins. This can cause inappropriate levels of gene expression in treated patients. In many cases, it is difficult or nearly impossible to predict when such unintended interactions will occur, unless empirical evaluation reveals the unexpected effects. Thus, a small plasmid that provides on the essential sequences is desirable.

Summary

The present invention is directed to novel plasmid DNA vectors that are substantially free of non-essential nucleotide sequences. Unnecessary or redundant DNA fragments are removed resulting in enhanced expression of the cloned gene in mammalian cells.

In one aspect of the invention there is a plasmid DNA vector consisting essentially of a replicon, and at least one other component selected from the group consisting of (i) promoter, (ii) intron, (iii) exogenous gene, (iv) transcription termination sequence, (v) selectable marker gene, and (vi) detectable marker gene wherein non-essential nucleotide sequences have been substantially removed.

In a second aspect of the invention there is provided a plasmid DNA vector consisting essentially of a promoter, origin of replication, polyadenylation signal, a bacterial resistance gene and a functional exogenous gene wherein the functional exogenous gene is operably linked to the promoter and wherein non-essential nucleotide sequences have been substantially removed.

In a further aspect of the invention there is provided a plasmid DNA vector consisting essentially of the following elements (i) a promoter functional in target cells, (ii) an intron, (iii) an exogenous gene, wherein said gene is operably linked to said promoter functional in target cells, (iv) a transcription termination sequence, (v) a replicon, and (vi) a selectable marker gene, wherein non-essential nucleotide sequences have been substantially removed.

In another aspect of the invention there is provided a plasmid DNA backbone consisting essentially of the following elements: (i) a promoter functional in target cells, (ii) an intron, (iii) a Multiple Cloning Site, wherein an exogenous gene may be inserted and operably linked to said promoter functional in target cells, (iv) a transcription termination sequence, (v) a replicon, and (vi) a selectable marker gene, wherein non-essential nucleotide sequences have been substantially removed.

In yet another aspect there is provided a host cell transfected with the inventive plasmid.

In a further aspect of the invention there is provided a method of transfecting a cell comprising contacting a cell with the inventive plasmid either *in vitro* or *in vivo* such that the plasmid enters the cell.

In another further aspect of the invention there is provided a method of gene therapy, either *in vivo* or *ex vivo*, comprising the administration to a patient in need of gene therapy a therapeutically effective amount of the inventive plasmid comprising an exogenous gene that is expressed in the patient.

Description of the Figures

Figure 1 is a representation of the novel plasmid DNA vector, peGT. Regions denoted G and H are sites for insertion of optional immunostimulatory sequences (ISS's). There may be greater than one, preferably two, ISS present.

Figure 2 is a representation of the novel plasmid DNA vector, peGT2.8, that does not contain the β-*galactosidase* gene. Regions denoted G and H are sites for insertion of optional immunostimulatory sequences (ISS's). There may be greater than one, preferably two, ISS present.

Figure 3 is a representation of the novel plasmid DNA vector, peGT2.6, that does not contain the SV40 intron nor the β-*galactosidase* gene. Regions denoted G and H are sites for insertion of optional immunostimulatory sequences (ISS's). There may be greater than one, preferably two, ISS present.

Figure 4 is a representation of the pVAX1/lacZ plasmid highlighting the regions where non-essential nucleotide sequences have been removed in the novel plasmids described herein. Regions denoted A (51 base pairs), B (114 base pairs) and C (112 base pairs) are non-essential nucleotide sequences which have been removed in the novel plasmids.

Figure 5 is a representation of the pCMVβ plasmid highlighting the regions where non-essential nucleotide sequences have been removed in the novel plasmids described herein. Regions denoted D (377 base pairs), E (157 base

pairs) and F (26 base pairs) are non-essential nucleotide sequences which have been removed in the novel plasmids.

Figure 6 is a graphical representation of Example 2. β-Gal expression in NIH3T3 cells by the novel vectors is compared with other commercially available vectors. β-Galactosidase activity of cells transfected with different plasmids using TransFast™ Reagent and grown in 6-well plates was assessed. Cell lysates were harvested 48 hours after transfection for β-galactosidase and protein assays. Expression of β-galactosidase was measured in milli-units of activity normalized by the amount of protein in each sample. The results represent the mean of triplicates; the error bars show the standard deviations. 1: non-transfected, 2&3: commercially available plasmids, 4: peGT, 5: peGT-ISS2, 6: peGT-ISS4.

Detailed Description

The invention will now be described in detail by way of reference only using the following definitions and examples. All patents and publications referred to herein are expressly incorporated by reference.

Definitions

As used herein, the following terms or abbreviations, whether used in the singular or plural, will have the meanings indicated:

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

A "detectable marker" gene is a gene that allows cells carrying the gene to be specifically detected (i.e., to be distinguished from cells that do not carry the marker gene). A large variety of such marker genes are known in the art. Preferred examples of such marker genes encode proteins appearing on cellular

surfaces, thereby facilitating simplified and rapid detection and/or cellular sorting. Also preferred are markers that allow visible detection of cells that carry the gene. Exemplary markers include, but are not limited to, a chemiluminescent, or enzymatic moieties.

A "selectable marker" gene is a gene that allows cells carrying the gene to be specifically selected for or against, in the presence of a corresponding selective agent. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows a host cell to be positively selected for in the presence of the corresponding antibiotic. A variety of positive and negative selectable markers are known in the art, some of which are described herein. In a preferred embodiment the selectable marker is a bacterial resistance gene such as kanamycin, ampicillin, tetracycline, chloramphenicol and the like.

"Heterologous gene" means a nucleic acid molecule or gene sequence that is (1) normally found in the targeted cells, (2) normally found in targeted cells but not expressed at physiologically appropriate levels in targeted cells, (3) normally found in targeted cells but not expressed at optimal levels in certain pathological conditions, (4) not normally found in the targeted cells, (5) novel fragments of genes normally expressed or not expressed in targeted cells, (6) synthetic modifications of genes expressed or not expressed within targeted cells, (7) any other DNA which may be modified for expression in targeted cells and (8) any combination of the above. Usually the heterologous gene is a contiguous fragment of DNA. The genetic material that is introduced into targeted cells can be any DNA. The heterologous gene encodes an epitope of interest, a biological response modulator, a growth factor, a recognition sequence, a therapeutic gene, a fusion protein or combinations thereof. The term heterologous gene may be used interchangeably with transgene, foreign gene, or exogenous gene.

"Immunostimulatory sequence" (ISS) means a nucleotide sequence that enhances the immune response of a DNA vaccine. Preferred sequences include,

but are not limited to, the hexamers GACGTC, AGCGCT, and especially AACGTT.

"Intron" means a non-coding sequence of DNA within a gene, i.e., an intervening sequence in the gene. It is transcribed into hnRNA but is then removed by RNA splicing in the nucleus, leaving a mature mRNA that is then translated in the cytoplasm. Introns are essential for normal expression of several mammalian genes. The inclusion of introns in the transcribed region enhances expression by an unspecified post-transcriptional mechanism. Introns may also contain transcriptional enhancers and thus enhance expression via a transcriptional mechanism.

"Multiple Cloning Site" means a synthetic DNA sequence encoding a series of restriction endonuclease recognition sites. These sites are engineered for convenient cloning of DNA into a vector at a specific position.

"Non-essential nucleotide sequence" means non-functional DNA sequences in a vector. These sequences are not required for the expression of the cloned gene, stabilization of transcribed products or plasmid replication. Other terms that may be used interchangeably herein to describe these sequences include unnecessary, superfluous, expendable, unneeded, unwanted, dispensable, immaterial or redundant.

"Promoter" means a region containing specific DNA sequences to which RNA polymerase binds before initiating the transcription of DNA into RNA.

"Replicon" means a DNA molecule that is able to initiate its own replication. A replicon must have an origin of replication and usually also has the necessary regulatory information required for the proper initiation of DNA replication. Plasmid copy number is one of the more important factors affecting plasmid DNA yield. Copy number is determined primarily by the replicon. Plasmids derived from pBR322 contain the ColE1 origin of replication from pMB1. This origin of replication is tightly controlled, resulting in approximately 25 copies of the plasmid per bacterial cell (low copy number). Plasmids derived

from pUC contain a mutated version of the ColE1 origin of replication, which results in reduced replication control and approximately 200-700 plasmid copies per cell (high copy number).

The term "substantially" should be given its ordinary and accustomed meaning, i.e., considerable, ample, large. Therefore, the phrase "substantially removed" means that non-essential sequences have been removed to a considerable, ample or large extent.

"Targeted cells" means either cells maintained, or propagated, in vivo or in vitro and capable of being transformed by plasmids as discussed herein.

"Transcription termination signal" means nucleotide sequences that function to stop transcription.

The inventive plasmid may be described by reference to its component elements. In the present invention, specific elements can be used to provide functionalities, e.g., plasmid copy number in bacteria and the ability to select plasmid-bearing bacterial cells, to a plasmid for delivery of nucleic acid and thus provide functionalities within a transformed cell or animal containing such a plasmid. Various plasmid elements have a variety of effects that influence plasmid use as gene therapy vectors. This includes the relative position and orientation of plasmid elements. Those skilled in the art will recognize that specific portions of these regions or elements can be identified as containing the functional aspects providing the desirable property. Such elements can be readily defined using routine techniques or their equivalents.

Component elements of the plasmid include, but are not limited to, a promoter, detectable marker gene, selectable marker gene, transcription termination signal, replicon, and intron. The inventive plasmid may contain genetic sequences coding for elements to assist in the purification of an expressed protein, e.g., His tags. The inventive plasmid will minimally comprise a replicon. The other components can be added or removed, depending on the purpose for which the plasmid will be used. For example, the promoter can be changed or

even deleted. A plasmid without a promoter can be used for determining the promoter activity of either non-naturally occurring synthetic sequences or naturally occurring sequences that may be cloned in. Similarly, the plasmid may contain no heterologous gene. In a preferred example, the inventive vectors of SEQ ID Nos 4-9 do not contain any functional gene. These vectors are designed to allow cloning any desired gene(s) into the vector. Likewise, the intron or polyA tail may be omitted to allow the insertion of another intron or polyA tail sequence, respectively. In some cases even the selection gene can be deleted. For example, the plasmid can be co-transformed with another plasmid, which will possess a selectable marker gene. Other selection methods utilizing the host's existing selectivity may also be used.

Additionally, any component may have more than one copy present in the plasmid. For example, one plasmid can have more than one functional gene, or contain more than one selectable marker genes.

The components may be in any orientation, any combination, and any order. For example, the beta-Gal gene, in plasmids of SEQ. ID Nos 1-3, is oriented 5' to 3', while the kanamycin resistance gene is oriented 3' to 5'.

The regulatory elements necessary for gene expression of a DNA molecule include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression. It is necessary that these elements be operably linked to the exogenous gene sequence that encodes the desired proteins when these elements are not already associated with the exogenous gene sequence to be inserted and that the regulatory elements are operable in the cell in which they are transfected.

Initiation codons and stop codons are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the cell in which the gene construct is transfected. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual. Examples of promoters useful to practice the present invention may be either prokaryotic or eukaryotic and include, but are not limited to, promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatinine and human metalothionein. Preferred embodiments include, but are not limited to, the eukaryotic promoters EF-1a, SV40, RSV, and to the prokaryotic promoters T7 , T3, SP6, EM7 and lac. The promoter may optionally be replaced by a Multiple Cloning Site for detection of endogenous promoter activity of the inserted exogenous DNA fragment.

Polyadenylation signals which may be positioned at the 3' end of the transgene include, but are not limited to, both synthetic or natural signals such as those derived from bovine growth hormone (BGH) and SV40. Examples of polyadenylation signals useful to practice the present invention include, but are not limited to, SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal is preferred.

The use of an intron positioned into a transcription unit, i.e., the exogenous gene, of the invention in order to activate the splicing apparatus and enhance message stability is within the scope of the invention.

The novel plasmid DNA vectors are constructed to substantially remove non-essential nucleotide sequences. With reference to pUC18 and two commercially available plasmid vectors, using the conventional numbering of the base pairs in the vectors, the inventive plasmids have the following characteristics:

(1) When compared to the commercially available plasmid pVAX1/LacZ (Invitrogen; SEQ ID NO. 10), the novel plasmid DNA vectors have removed the

non-coding region before the pUC origin of replication. The 51 base pairs corresponding to 5092-5143 of pVAX1/LacZ (equivalent to base pairs 449-500 of pUC18, which is the 3' non-coding region of the lacZ gene in pUC18) were removed. The novel plasmid DNA vectors have also removed the unnecessary ampicillin promoter before the pUC origin of replication corresponding to the base pairs 5144-5258 of pVAX1/LacZ (equivalent to base pairs 754-868 of pUC18). The pVAX1/LacZ vector is a kanamycin resistant vector; the ampicillin promoter is non-essential. Additionally, the unnecessary region before the CMV promoter of pVAX1/LacZ corresponding to base pairs 1-112 has been removed. See Figure 4.

(2) When compared to the commercially available plasmid pCMVβ (Clontech; SEQ ID NO. 11), the novel plasmid DNA vectors have removed the 377 base pairs corresponding to 4513-4890 of pCMVβ (the lacZ promoter of pUC18) and the 157 base pairs corresponding to 7007-7164 of pCMVβ (the lacZ 5' coding region in pUC18). pUC18 contains a simple version of the lacZ gene different from the large lacZ gene in other vectors. The small pUC18 lacZ is redundant in the pCMVβ vector. Therefore, any fragments belonging to it were removed. Additionally, the unnecessary region before the CMV promoter of pCMVβ corresponding to base pairs 1-26 has been removed. See Figure 5.

The inserted heterologous gene in a plasmid of the present invention, preferably encodes, but is not limited to, an expression product comprising: an epitope of interest, a biological response modulator, a growth factor, a recognition sequence, a therapeutic gene or a fusion protein.

Expression of the heterologous gene in a suitable host cell, e.g., prokaryotic or eukaryotic cells, allows for the production of large quantities of the expressed gene product, i.e. polypeptide or protein. A host cell strain for use with the inventive plasmids may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage or

folding) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems are well known in the art and can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to 3T3, CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc. and selection of the appropriate cell line is within the ambit of one skilled in the art. Yeast systems are useful for expression and analysis of eukaryotic proteins. Insect cells are useful for recombinant protein expression and production.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the heterologous gene may be engineered. Following the introduction of the novel plasmids by methods known in the art (for example $CaPO_4$ precipitation, electroporation and the like), engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the novel plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the desired heterologous gene on the cell surface, and are particularly useful in screening analogs for the gene product of the heterologous gene. This method is also suitable for the production of secreted polypeptides that may encoded by heterologous genes.

The present inventive DNA vectors also find use in gene therapy, i.e., the transfer of selected genes into a host with the hope of ameliorating or curing a disease state. The inventive plasmids replace an absent or faulty gene with a

working gene, so that the body can make the correct enzyme or protein and consequently eliminate the root cause of the disease. The working gene is a heterologous gene obtained from an exogenous source.

Gene therapy for genetically caused diseases, such as cystic fibrosis or muscular dystrophy, is based on the discovery that such diseases are caused by particular genetic defects. A therapy for the genetic defect may be possible if the healthy gene is supplied to the afflicted organism in a sufficient amount. Gene therapy not only enables the treatment of genetically caused diseases, but is also suitable for the treatment of tumors, and is suited as a new form of inoculation against infectious diseases, such as hepatitis, influenza, and HIV, to give but a few examples.

The gene therapies according to the present invention comprise about 1 picogram to about 1000 milligrams of DNA. The therapy may be localized to a specific region or organ, or it may be administered systemically. In some preferred embodiments, the therapy contains about 1 nanograms to about 1000 micrograms of DNA. In some preferred embodiments, the therapy contains about 0.1 micrograms to about 500 micrograms of DNA. In some preferred embodiments, the therapy contains about 1 to about 350 micrograms of DNA. In some preferred embodiments, the therapy contains about 25 to about 250 micrograms of DNA. In some preferred embodiments, the therapy contains about 100 micrograms DNA.

Any of the methods for gene therapy available in the art can be used according to the present invention. For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, *TIBTECH* 11(5):155-215). Methods commonly known in the art of recombinant DNA technology for selecting and cloning the heterologous gene are described in Ausubel et al. (eds.),

1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, N.Y.

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of the heterologous gene mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

The inventive plasmids described herein may comprise DNA sequences coding for polypeptides that have useful therapeutic applications. These inventive plasmids may be naked in the sense that they are free from any delivery vehicle that can act to facilitate entry into the cell, for example, the polynucleotide sequences are free of viral sequences, particularly any viral particles which may carry genetic information. Similarly, they may be free from, or naked with respect to, any material which promotes transfection, such as liposomal formulations, charged lipids such as Lipofectin™ or precipitating agents such as $CaPO_4$. However, it is within the scope of the invention that the inventive plasmids may be used in conjunction with other agents such as, for example, those listed above to enhance DNA delivery and transfection.

The genetic vaccines according to the present invention comprise about 1 picogram to about 1000 milligrams of DNA and a maximal dose of 10 grams is contemplated. In some preferred embodiments, the vaccines contain about 10

picograms to about 800 milligrams of DNA. In some preferred embodiments, the vaccines contain about 0.1 to about 500 milligrams of DNA. In some preferred embodiments, the vaccines contain about 1 to about 350 milligrams of DNA. In some preferred embodiments, the vaccines contain about 25 to about 250 milligrams of DNA. In some preferred embodiments, the vaccines contain about 100 milligrams DNA. However, it is within the skill of art to appropriately adjust the amount of DNA to the response desired. Multiple doses may be required and/or administered.

The genetic vaccines according to the present invention are formulated according to the mode of administration to be used. One having ordinary skill in the art can readily formulate a genetic vaccine that comprises a novel plasmid described herein. In cases where intramuscular injection is the chosen mode of administration, an isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free.

In some cases, the use of ISS's may be desirable to enhance the genetic vaccines. Currently, there are no ISS containing vectors commercially available. The present inventive plasmids may contain zero or multiple ISS's. The presence of the ISS's may be without adverse effect on the expression of inserted exogenous heterologous gene. ISS's are palindromic DNA sequences that induce production of interferon and enhance natural killer cell activity. However, it has been noted that the ISS's may interfere with gene expression. See Sato et al. Science (1996) 273:352-354. Surprisingly, the ISS's in the inventive plasmids have not been found to interfere with gene expression.

The following are examples of the present invention using various plasmid elements to design and construct plasmids for delivery of nucleic acid to various tissues. In particular, the following are specific examples of the construct peGT

and its derivatives. The utility of the plasmids of the present invention is noted by use in nonviral human gene therapy, genetic vaccines, cloning and expression of genes, and other standard molecular biology uses.

Examples

The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

Example 1

Plasmid Construction

A plasmid containing the lacZ gene was constructed. A schematic of the inventive plasmid constructed and utilized in subsequent experiments is shown in Figure 1, corresponding to SEQ ID NO 1. The plasmid contains the following elements: a promoter, a splice site, the lacZ gene, a transcription termination signal, a replicon, and bacterial resistance gene. In other embodiments, the bacterial resistance gene may be flanked with zero, one (SEQ ID NO 2) or two (SEQ ID NO 3) copies of an ISS on each side of the gene. The plasmid vectors used in the experiments described below were constructed using the CMV promoter, SV40 intron, the lacZ gene, the SV40 polyadenylation sequence, pUC origin of replication, and the kanamycin resistance gene with either zero, one or two copies of an ISS on each side. Plasmids without an ISS on each side of the kanamycin resistance gene are designated peGT. Plasmids with one ISS copy on each side of the kanamycin resistance gene are designated peGT-ISS2. Plasmids with two ISS copies on each side of the kanamycin resistance gene are designated peGT-ISS4.

Example 2

Plasmid Expression

Expression of the lacZ gene incorporated into three inventive plasmid vectors (peGT, peGT-ISS2 and peGT-ISS4) described in Example 1 was compared with two commercially available plasmids, pCMVβ (Clontech Laboratories, Palo Alto, CA 94303) and pVAX1/LacZ (Invitrogen Corporation, Carlsbad, CA 92008) in NIH3T3 cells. Surprisingly, the inventive plasmids, despite their smaller size, did not have a reduced level of expression, but rather attained a higher level of expression.

Cells were plated one day before transfection at the density of $2.5x10^5$ cells/well in 6-well plates. Cells were transfected using TransFast™ Reagent (Promega, Madison, WI). The total volume of medium, DNA, and TransFast™ Reagent (Promega, Madison, WI) to add per well for 6-well plates was 1 ml. On the day of transfection, the appropriate amount of serum-free medium (pre-warmed to 37°C) was added to a sterile tube. 2.5 μg of plasmid DNA/well were added to the medium and vortexed, and 3 μl of TransFast™ Reagent (1mM)/μg of DNA (1:1 charge ratio of TransFast™ Reagent to DNA) were then added and vortexed immediately. For each plasmid, 3 wells of 6-well plates were used. The DNA/TransFast™ Reagent/medium mixtures were incubated for 15 minutes at room temperature. Growth medium was removed from the cells, and the DNA/TransFast™ Reagent/medium mixtures were briefly vortexed and added to each well (1ml/well). Cells were returned immediately to the 37°C incubator for 1 hour. At the end of the incubation period, cells were gently overlayed with 2 ml of complete media and were returned to the 37°C incubator for 48 hours.

β-galactosidase activity was measured using β-galactosidase Enzyme Assay System (Promega, Madison, WI). Cells were harvested 48 hours after transfection. Cells from each well of 6-well plates were washed twice with PBS (phosphate-buffered saline, 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.47mM KH_2PO_4), and lysed with Reporter Lysis Buffer (200μl for each well).

After 15-minute incubation at room temperature, cells lysate was scraped, and collected into a microcentrifuge tube on ice. Cell lysate was then vortexed and centrifuged at 14,000xg, and the supernatant was transferred to a fresh tube. Adequate amount of cell extracts were taken and mixed with Reporter Lysis Buffer to 50µl, and 50µl of Assay Buffer was added. The mixtures were incubated at 37°C for 30 minutes, and the reactions were stopped by 150 µl of 1M sodium carbonate. β-galactosidase activities were determined by the absorbance reading at 420nm in a 96-well plate.

Protein amounts were determined by BCA Protein Assay Kit (Pierce, Rockford, IL) using BSA (bovine serum albumin) as standards. β-galactosidase activity was normalized by protein quantity in each well.

The novel plasmids were efficiently transfected into and demonstrated a high level of gene expression in the host cells. The inventive plasmids demonstrated at least a two-fold increase in β-galactosidase activity per milligram protein when compared to commercially available plasmids. See Figure 6. Compared to the pCMVβ plasmid (Column 2, Figure 6), the inventive plasmid peGT expressed greater than two times more β-galactosidase activity. Similarly, when compared to the pVAX1 plasmid (Column 3, Figure 6), the inventive plasmid peGT expressed about ten times more β-galactosidase activity. Thus, despite their smaller size the inventive plasmid expressed higher levels of the desired gene product.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

1. A plasmid DNA vector consisting essentially of a replicon, and at least one other component selected from the group consisting of:

(i) promoter,

(ii) intron,

(iii) exogenous gene,

(iv) transcription termination sequence,

(v) selectable marker gene,

(vi) detectable marker gene and

(vii) an immunostimulatory sequence

wherein non-essential nucleotide sequences have been substantially removed.

2. A plasmid according to Claim 1 consisting essentially of a replicon and an exogenous gene.

3. A plasmid according to Claim 1 consisting essentially of a replicon, an exogenous gene and a selectable marker gene.

4. A plasmid according to Claim 1 consisting essentially of a replicon, an exogenous gene, a selectable marker gene and a promoter.

5. A plasmid according to Claim 1 wherein the exogenous gene is a detectable marker gene.

6. A plasmid DNA vector consisting essentially of a promoter, origin of replication, polyadenylation signal, a bacterial resistance gene and a functional exogenous gene wherein the functional exogenous gene is operably linked to the

promoter and wherein non-essential nucleotide sequences have been substantially removed.

7. A plasmid DNA vector consisting essentially of the following elements:

(i) a promoter functional in target cells,

(ii) an intron,

(iii) an exogenous gene, wherein said gene is operably linked to said promoter functional in target cells,

(iv) a transcription termination sequence,

(v) a replicon, and

(vi) a selectable marker gene,

wherein non-essential nucleotide sequences have been substantially removed.

8. A plasmid according to Claim 7 wherein the exogenous gene encodes a detectable marker.

9. A plasmid according to Claim 7 wherein the selectable marker is a bacterial resistance gene.

10. A plasmid DNA backbone consisting essentially of the following elements:

(i) a promoter functional in target cells,

(ii) an intron,

(iii) a Multiple Cloning Site, wherein an exogenous gene may be inserted and operably linked to said promoter functional in target cells,

(iv) a transcription termination sequence,

(v) a replicon, and

(vi) a selectable marker gene,

wherein non-essential nucleotide sequences have been substantially removed.

11. A host cell transfected with the plasmid of Claim 1.

12. A method of transfecting a cell comprising contacting a cell with said plasmid of Claim 1 *in vitro*, such that said plasmid enters said cell.

13. A method of transfecting a cell comprising contacting a cell with said plasmid of Claim 1 *in vivo*, such that said plasmid enters said cell.

14. A method of gene therapy comprising the administration to a patient in need of gene therapy a therapeutically effective amount of the plasmid of Claim 1 comprising an exogenous gene that is expressed in said patient.

15. A method of *ex vivo* gene therapy comprising the administration to a patient in need of *ex vivo* gene therapy a therapeutically effective amount of transfected cells comprising the plasmid of Claim 1 comprising an exogenous gene that is expressed in said cells and reintroducing said cells to said patient.

SEQUENCE LISTING

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Sun, Lihyun

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<211> 5943
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA plasmid

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<211> 2624
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA plasmid

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<211> 2636
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA
plasmid

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<210> 6
<211> 2648
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA
plasmid

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<210> 7
<211> 2821
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA plasmid

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g 2821

<210> 8
<211> 2833
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA plasmid

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atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc 180
aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta 240
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ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg gtaggcgtgt 480
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accgcatcag gcg 2833

<210> 9
<211> 2845
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:novel DNA plasmid

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atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc 180
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<210> 10
<211> 6050
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:commercial plasmid

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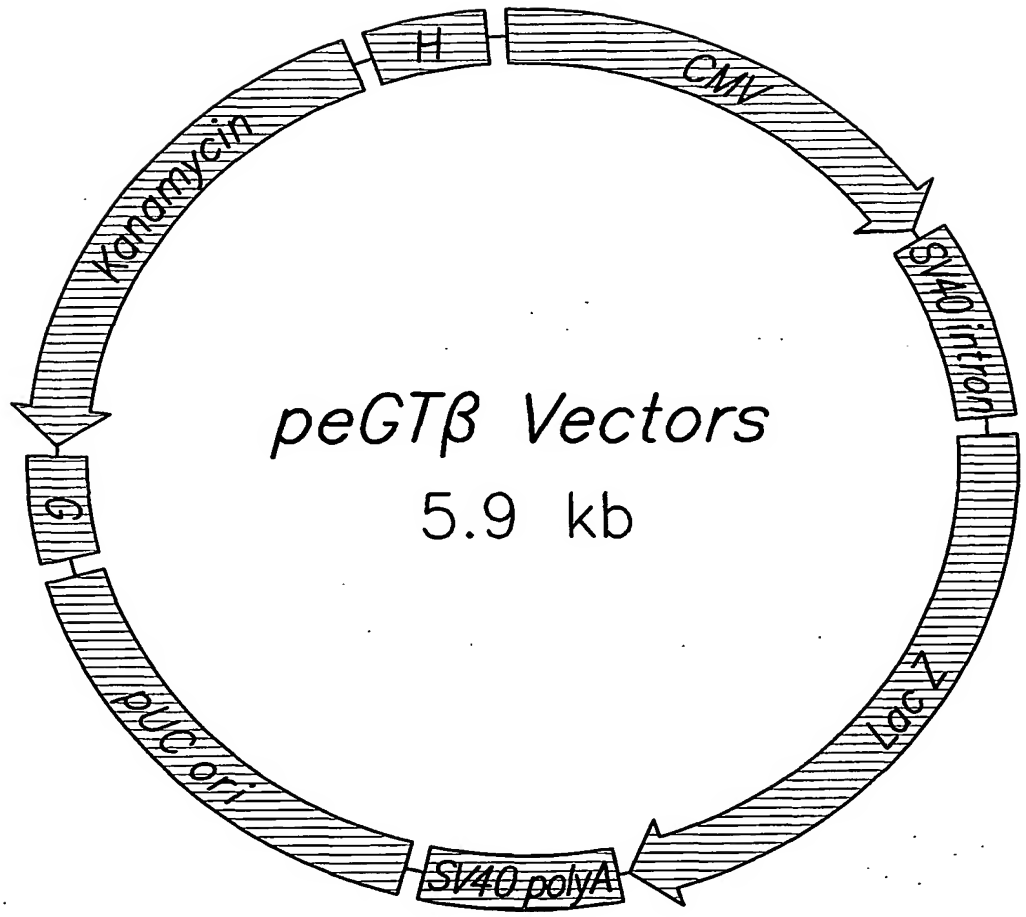

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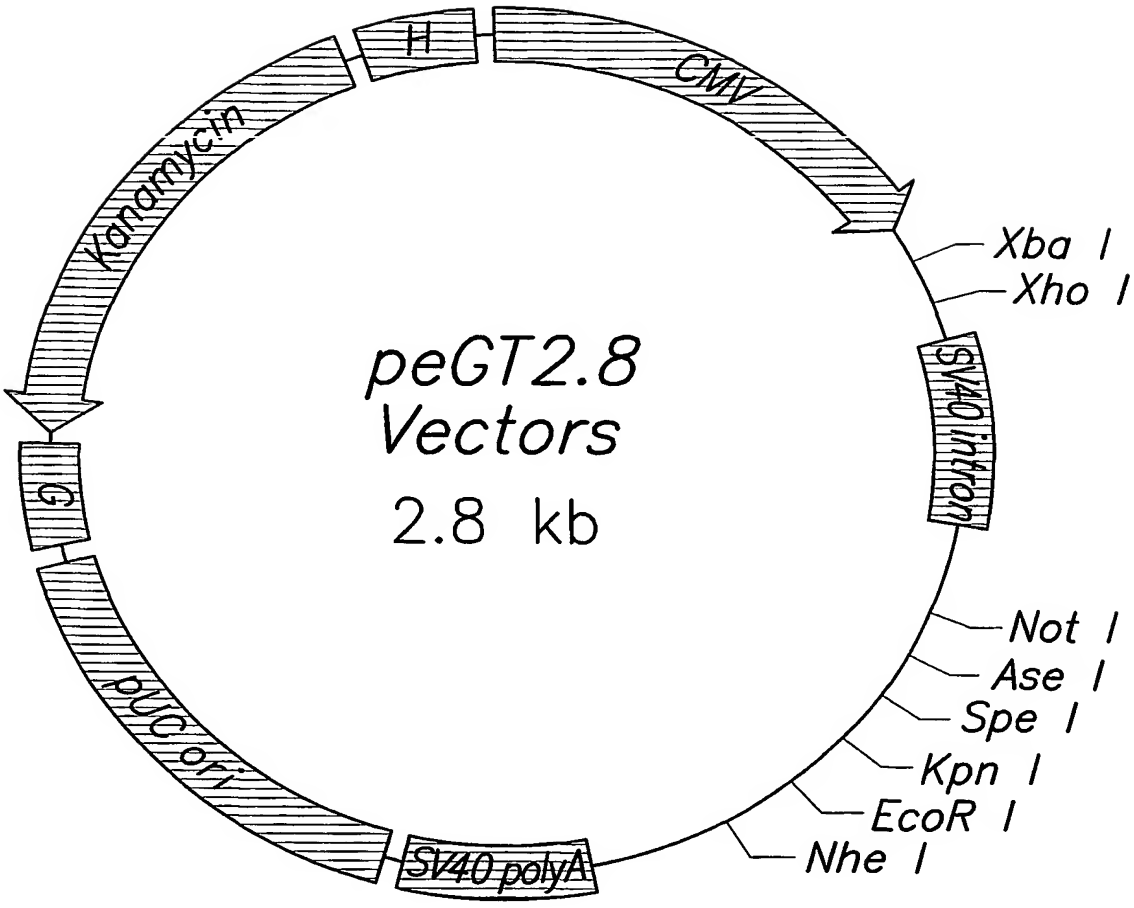

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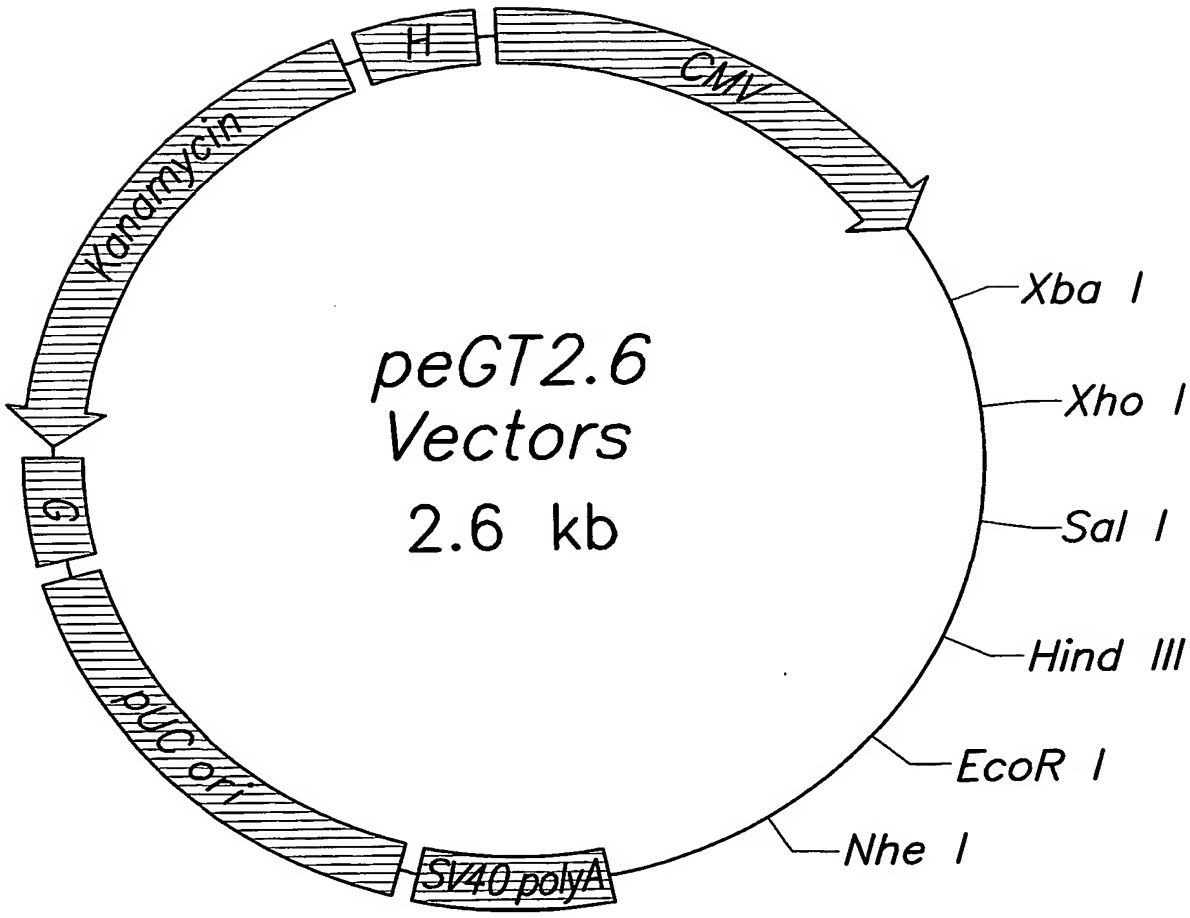

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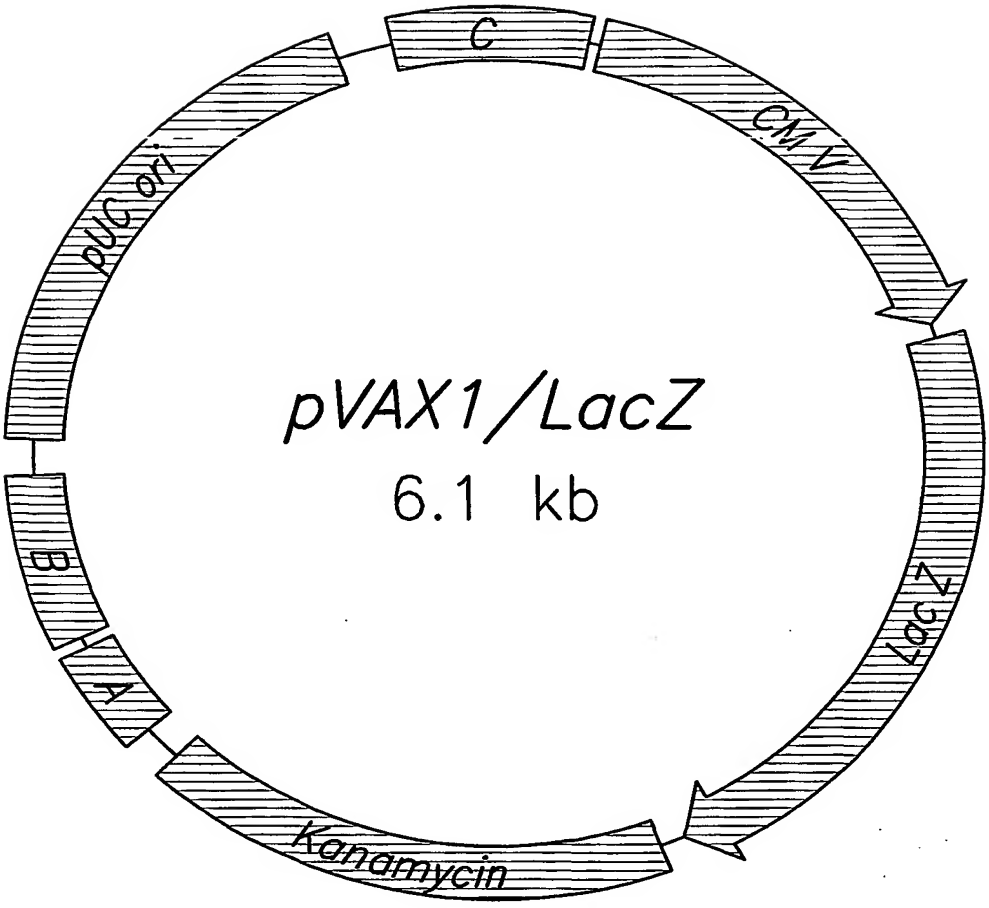
FIG. 3



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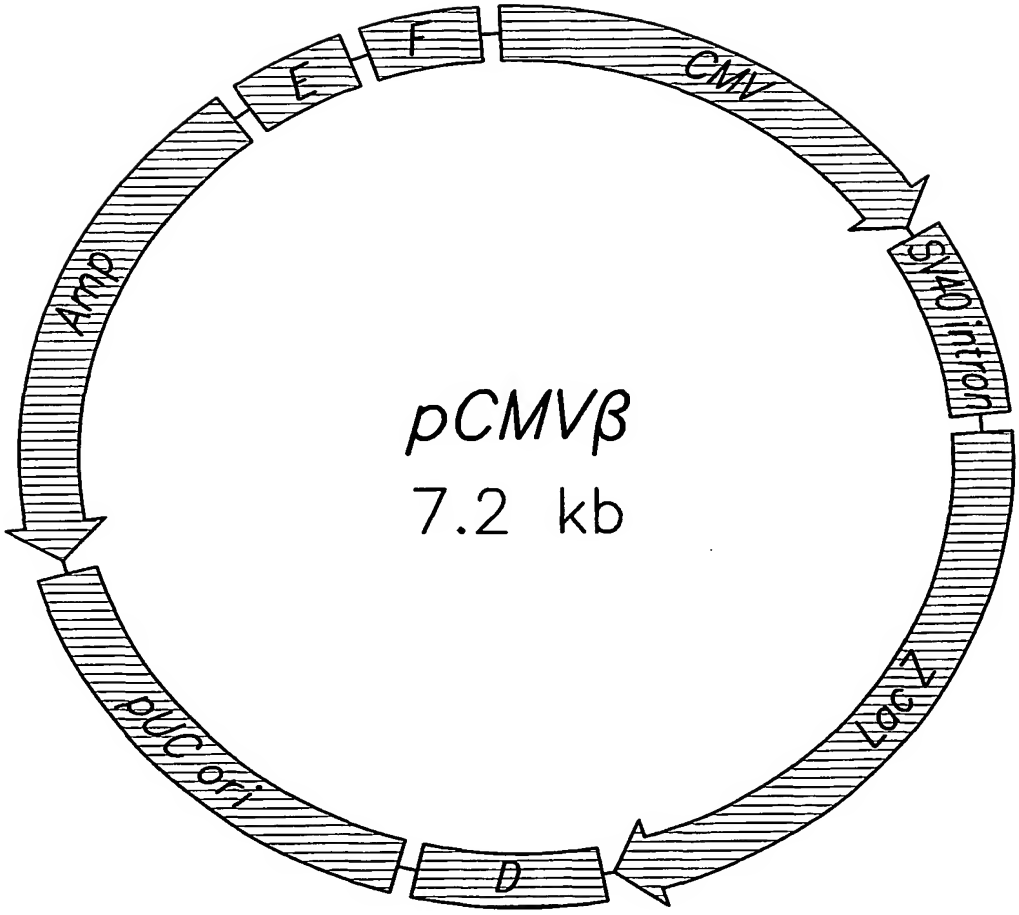

FIG. 5

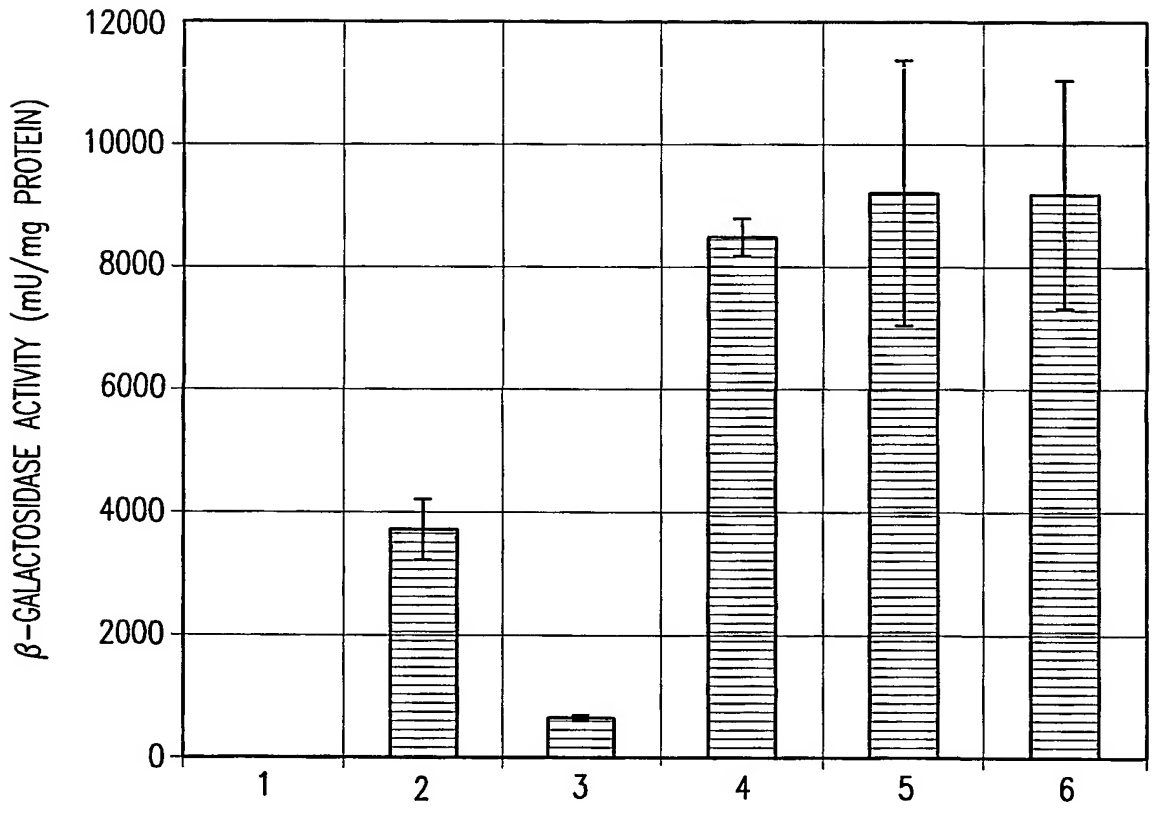

FIG. 6

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(71) Applicant: ELIM BIOPHARMACEUTICALS, INC. [US/US]; 901 Grandview Drive, South San Francisco, CA 94080 (US).

(72) Inventors: LU, Xianbin; 311 Faxon Avenue, San Francisco, CA 94112 (US). SUN, Lihyun; 140 Ware Road, Woodside, CA 94062 (US). ZHANG, Yilin; 140 Ware Road, Woodside, CA 94062 (US).

(74) Agents: CHOW, Y., Ping et al.; Heller Ehrman White & McAuliffe LLP, 275 Middlefield Road, Menlo Park, CA 94025-3506 (US).

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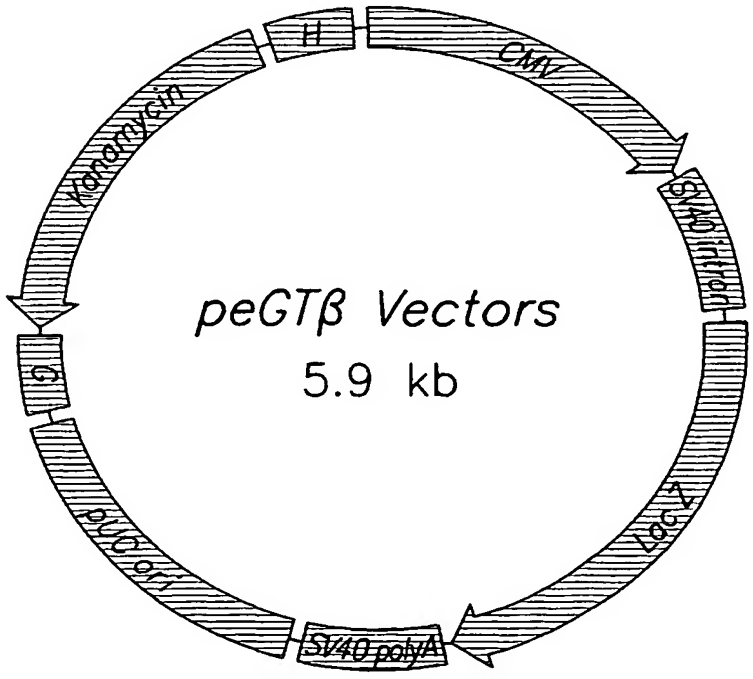
[Continued on next page]

(54) Title: NOVEL PLASMID EXPRESSION VECTORS

(57) Abstract: This invention relates to plasmid backbones and constructs wherein non-essential nucleotide sequences have been substantially removed. These backbones can be used to clone genes of interest. The constructs are useful in gene therapy, DNA vaccines and the *in vitro* production of polypeptides and/or proteins.



peGT β: G=H=0
peGT β -ISS2: G=H=1 ISS
peGT β -ISS4: G=H=2 ISS

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(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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INTERNATIONAL SEARCH REPORT

Inte tional Application No
PC1/US 01/01255

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 C12N15/67 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARTIKKA J: "AN IMPROVED PLASMID DNA EXPRESSION VECTOR FOR DIRECT INJECTION INTO SKELETAL MUSCLE" HUMAN GENE THERAPY, vol. 7, no. 10, 20 June 1996 (1996-06-20), pages 1205-1217, XP002050079 ISSN: 1043-0342	1-14
Y	page 1206, left-hand column; table 1 page 1210, right-hand column -page 1211, left-hand column, paragraph 2	15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 July 2001	31/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Steffen, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	page 779, left-hand column -page 780, left-hand column, paragraph 1; figure 1	14,15
X	INVITROGEN: "pVAX1 (Catalog no. V260-20) Version B 010124/25-0256" INVITROGEN HOMEPAGE (WWW.INVITROGEN.COM), 'Online! 1998 - 2001, XP002171177 Retrieved from the Internet: <URL:http://www.invitrogen.com/Content/Tech-Online/molecular_biology/manuals_pps/pvax1_man.pdf> 'retrieved on 2001-07-03!	1-6, 11-13
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X	LANG C ET AL: "Efficient expression and secretion of Aspergillus niger RH5344 polygalacturonase in Saccharomyces cerevisiae." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 44, no. 1-2, 1995, pages 147-156, XP001005274 ISSN: 0175-7598 page 150, left-hand column, paragraph 3 -page 151, right-hand column, paragraph 3; figure 2; table 1	1-5, 11-13

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	AZEVEDO V ET AL: "Main features of DNA-based immunization vectors." BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH, vol. 32, no. 2, February 1999 (1999-02), pages 147-153, XP002171178 ISSN: 0100-879X page 149 -page 152, right-hand column, paragraph 1; figure 1 ---	1-15
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Y	KAY M A ET AL: "GENE THERAPY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 94, November 1997 (1997-11), pages 12744-12746, XP002922034 ISSN: 0027-8424 page 12744, left-hand column, paragraph 3 page 12476, left-hand column ---	14,15
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A	CLONTECH: "Vector Info pCMVbeta" CLONTECH HOMEPAGE (WWW.CLONTECH.COM), ‘Online! 1997, XP002171179 Retrieved from the Internet: <URL:http://www.clontech.com/techinfo/vectors/vectorsC/pCMVbeta.shtml#description> ‘retrieved on 2001-07-03! the whole document	
A	-& CLONTECH: "pCMVbeta Vector Information" CLONTECH HOMEPAGE (WWW.CLONTECH.COM), ‘Online! 1997 - 1997, XP002171180 Retrieved from the Internet: <URL:<URL:http://www.clontech.com/techinfo/vectors/vectorsC/pdf/PT2004-5.pdf>> ‘retrieved on 2001-07-03! the whole document	
P,X	WO 00 40737 A (KIM DUK KYUNG ;LEE YOUNG JOO (KR); YU SEUNG SHIN (KR); KIM SUNYOUN) 13 July 2000 (2000-07-13) page 19, line 12 -page 21, line 20; figures 1,4-6	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/01255

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0040737 A	13-07-2000	AU 1896500 A	24-07-2000